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ENHANCED RECOVERY OF COLIFORMS BY ANAEROBIC INCUBATION

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16. ABSTRACT

A total of 529 well and distribution potable water samples were analyzed for total coliforms by the most probable number (MPN) and membrane filter (MF) techniques. Standard plate count (SPC) bacteria and MF non-coliform bacteria were also enumerated. Frequency of coliform detection, turbidity in MPN tubes and extensive overgrowth by non-coliforms on MF filters were directly proportional to SPC. Overgrowth by non-coliforms on MF filters suppressed sheen development and, in turn, masked coliform detection. Of 341 atypical (no-sheen) MF colonies verified by parallel inoculation of lauryl sulfate and brilliant green broth, 156 were aerogenic in the latter medium. Of atypical isolates, 84% were identified as species of Citrobacter and Enterobacter. A 4.4-fold reduction in numbers of overgrown MF filters and a 2.2-fold increase in numbers of coliforms recovered from 127 water samples was accomplished by anaerobic incubation of MF cultures. This anaerobic modification of the standard MF technique significantly reduced overgrowth and enhanced recovery of coliforms from potable groundwater.

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ENHANCED RECOVERY OF COLIFORMS BY ANAEROBIC INCUBATION

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The sanitary quality of drinking water is, in part, determined by the presence of total coliform bacteria, and Federal Regulations state that total coliform analyses be performed by the most probable number (MPN) or membrane filter (MF) procedures (1,3). The MF procedure is preferred since it is more quantitative and larger volumes of sample can be readily assayed (5,8,11)

Accumulated evidence reviewed recently by Evans et al (6,7) and Means and Olson (17) suggests that recovery of coliforms by both of these procedures is subject to interference by non-coliform microorganisms, and several explanations have been offered.

With the MF procedure, those factors that suppress growth of coliforms or sheen development by coliform colonies would result in a significant overestimation of drinking water quality. Data obtained from a survey of community water supply systems (CWSS) (14) analyzed by Geldreich et al (9,10) showed that the frequency with which coliforms were detected by the MF procedure was not adversely affected when SPC densities did not exceed 500/mL, but decreased as the SPC densities exceeded 1000/ml.

Although the nature of suppression of coliform detection was not defined, results of subsequent investigations suggest that overgrowth on MF membranes by non-coliforms either inhibits coliform growth or suppresses sheen formation (7,11). Indeed, failure to recognize and verify atypical (non-sheen) coliforms in the MF procedure has been implicated in significant underestimation of coliform densities in drinking water. The need for control of SPC populations and their role in deterioration of potable water quality has been addressed by Geldreich et al (9,10) and others (12). Population densities and types of SPC bacteria in potable water samples not only reflect the quality of the source water, but also reflect the efficacy of its treatment, disinfection and distribution. Groundwater is often distributed without treatment or disinfection. In the CWSS survey cited above, 67% of well waters were distributed to customers without disinfection. Public water supplies in Southern Arizona are derived primarily from ground sources and are neither treated nor routinely disinfected prior to distribution. The present study was undertaken to assess the bacteriological quality of both source (well) and distribution water samples and to identify specific problems associated with recovery of coliforms from these samples containing SPC bacteria.

MATERIALS AND METHODS

Description of the distribution system. The Tucson Water Utility is the major supplier of drinking water to the Tucson metropolitan area and provides service to approximately 450,000 people through 130,000 service connections. Water is derived solely from wells which range in depth from approximately 250 ft. to 1200 ft. and, in most cases, is neither treated nor disinfected prior to distribution.

Water sample sources and collection. A total of 220 well and 309 distribution water samples were collected between January, 1981 to January, 1983 from 201 wells and 250 distribution sites. Sampling sites were representative of those used by the Tucson Water Department for routine microbiological sampling. Water samples were collected in sterile 1 liter polypropylene bottles with added sodium thiosulfate. Water temperature was recorded at time of sampling and the samples were transported on ice packs to the laboratory and analyzed within 2 h after collection. Total coliforms were enumerated by the membrane filter technique (MF) according to established methods (1,3) for potable water unless otherwise indicated. Water samples (100 ml) were filtered through Gelman GN-6 membranes (pore size, 0.45 μm). Membranes were placed directly onto m-endo broth (ME; BBL) solidified with agar or m-endo LES agar (LES; Difco Laboratories). Both typical and atypical (red or pink with no sheen) colonies were picked and verified by simultaneous inoculation into LSB and BGB. In order to overcome potential masking of coliform detection on overgrown plates, i.e., too numerous to count (TN) or confluent growth (CG), an anaerobic MF (An-MF) technique was used. This involved incubating replicate MF plates anaerobically (Brewer anaerobic jar, BBL) containing a H_2-CO_2 generating system (Gas-Pak, PBL). To ensure anaerobic conditions the tight-fitting lids of MF plates were perforated after placement of the filter to allow gas exchange. This was accomplished by melting a hole in the sides of the dishes just below the top surface with a dissecting needle (0.5 mm diameter) heated to redness in a flame and cooled approximately 1 to 2 sec. In this manner four perforations were made. Perforating the sides of the dishes reduced the risk of contamination and also facilitated stacking in anaerobic jars. Background bacterial densities of all samples were determined by the standard plate count (SPC) procedure (1,3) using standard methods agar (SMA, BBL).

Isolation and identification of bacteria. Both typical and atypical coliform colonies picked from MF and EMB plates were purified by streaking plates of SMA. Purified colonies were identified by the API 20E method (18).

Quality control and statistical analyses. A quality assurance program as outlined (3) was followed throughout this study. Data were analyzed by analysis of variance (ANOVA).

RESULTS

Mean temperature of 220 well samples was 26°C and varied from 23°C to 27°C at time of collection. No seasonal vari-

ation was observed. Mean temperature of 309 distribution samples was 27°C, and ranged from 12°C to 35°C. Seasonal variation was observed. The mean temperature of 48 samples collected during the winter months of December, January and February was 20°C with a range 17°C to 23°C and the mean temperature of 84 samples collected during the summer months of June, July and August was 30°C with a range of 18°C to 35°C.

Coliforms were isolated from 31 of 529 well and distribution samples analyzed (Table 1) and detection of coliforms increased as SPC increased. Relative frequency of occurrence of overgrowth (TN/CG) of non-coliforms on MF plates also increased as SPC increased.

The frequency with which coliforms were isolated from randomly sampled wells was highest in July, 1981, a month in which rainfall exceeded normal expectation. Over 6 in. (15.57 cm) was recorded by the National Weather Service, Tucson, AZ. The mean SPC for 46 wells sampled was 9.96×10^1 ($\log_{10}$ 1.9982, 95% C.L. ± 0.1993) and 13 wells (28%) were coliform positive. Although only 0.8 in. (2.03 cm) rainfall was recorded in August, the SPC of 30 wells sampled did not decrease significantly and 5 (17%) were coliform positive. In contrast, the mean SPC of 48 well samples collected in June (0.41 cm rainfall) was significantly lower, i.e., 3.11×10^1 ($\log_{10}$ 1.4930, 95% C.L. ± 0.1950) and only one sample was coliform positive. Results were similar for 39 well samples collected in January, 1981. None of 84 distribution sites sampled in June, July and August of 1981 contained coliforms.

Coliform isolation and identification. A total of 453 typical and atypical colonies derived from 19 well and 12 distribution samples were picked and verified in parallel in both LSB and BGB broth. Of the 112 typical colonies picked, 86% verified in BGB, and 156 (46%) of 341 atypical colonies picked were verified. Of the 156 atypical colonies that verified in BGB, 55 were derived from 12 well samples that, in addition, produced overgrowth of MF plates by non-coliforms. Overgrowth suppressed sheen formation which, in turn, led to underestimation of coliform density. For example, the mean coliform density of only typical (sheen) colonies for these 31 samples by standard MF technique was 3.1 while the mean coliform density for both typical and atypical colonies was 8.1. The identification of 69 coliforms isolated from MPN analyses and 252 coliforms isolated from MF analyses are shown in Table 2. *E. cloacae* and *C. freundii* were the predominant species identified by both methods of enumeration.

Interference of coliform detection by MF. Overgrowth of MF plates by non-coliforms increased as SPC increased (Table 1). At SPC densities exceeding 500 cfu/ml, 18 of 37 samples produced overgrowth on filters and false negative reactions were observed. Fifty membrane filter cultures with excessive growth and no differentiated sheen colonies were transferred to LSB and observed for gas production after 48 h, 35°C incubation. Ten of the 50 confirmed in BGB broth. Since 7 of these samples were initially coliform

positive by the MPN method, development of confluent growth masked detection of coliforms.

Anaerobic MF. Both surface and groundwater contain bacteria considered antagonistic to coliforms (12,17). Since the majority of these are aerobic, their growth and, in turn, influence on recovery of coliforms by the MF method could be suppressed by anaerobic incubation. The influence of anaerobic incubation on recovery of environmental coliform isolates suspended in distilled water is shown in Table 3. With the exception of C. freundii, there was no significant difference ($p = 0.05$) between the two methods with respect to the numbers of sheen colonies counted at each holding time interval. Atypical colony formation by C. freundii became typical after an additional 24 h anaerobic incubation. Numbers of coliforms recovered decreased with holding time in all cases except K. pneumoniae. Growth of this organism in distilled water was subsequently confirmed. To evaluate further this modification of the standard MF technique, 127 water samples were tested by both standard aerobic (A-MF) and anaerobic (AN-MF) incubation of MF plates. For 101 samples (44 well and 57 distribution) m-Endo agar was used and for 26 samples (17 well and 9 distribution) m-Endo agar LES was used. A total of 105 coliforms were verified from 11 samples by the A-MF method and 236 coliforms were verified from 14 samples by the AN-MF method (Table 4). Of the samples tested, 17% were TN/CG by standard aerobic incubation while only 4% were TN/CG by anaerobic incubation (Table 5). Thus, irrespective of the plating medium, anaerobic incubation yielded a 2.2-fold increase in numbers of verified coliforms and a 4.4-fold reduction in numbers of overgrown MF filters. The effectiveness of anaerobic incubation in suppressing non-coliform background growth on MF filters is depicted in Figures 1 and 2. MF plates shown in Figure 1 were derived from filtration of 100 ml of well water that had an SPC of 5.9×10^3 cfu/ml, a turbidity of 9.2 NTU and an MPN index of >16. The aerobic plate (A) was overgrown by non-coliforms and two sheen colonies are evident on the lower left periphery. In contrast, the background of the anaerobic plate (AN) was clear and 18 sheen colonies are evident. The MF plates shown in Figure 2 were obtained from filtration of 100 ml of surface (stream) water that had an SPC of 3.9×10^3 cfu/ml, a turbidity of 3.3 NTU and an MPN index of 24. In this case also, the aerobic plate (A) is extensively overgrown and devoid of typical colonies. Well over 100 sheen colonies, however, are evident on the anaerobic plate (AN). Thus, anaerobic incubation effectively unmasked colifrom detection in both of these samples.

Holding time and temperature. Conditions under which water samples were held in the laboratory prior to analysis influenced bacteriological quality. For example, the mean ratios (to 0 hr) in SPC for 11 well and distribution samples held at 6°C for 6h, 24h, 30h and 48h were 0.684, 0.824, 0.668 and 0.621, respectively. In contrast, mean ratios in samples held at 21°C to 25°C for the same times were 1.71, 38.8, 74.9 and 277.7, respectively. Initial SPC ranged from 10^1 to 10^3 cfu/ml, final (48h) SPC ranged from 10^4 to

10^6 cfu/ml for all samples and MF cultures were extensively overgrown.

The influence of growth of bacteria in stored water on recovery of coliforms was investigated as follows. Replicate 2 L well water samples were collected in sterile polypropylene bottles with added thiosulfate. Samples stored at 6°C and 27°C were assayed for total coliforms (A-MF and AN-MF, 100 ml) and SPC at 0h, 24h, 30h, 48h and 54 h, respectively. As shown in Table 6, storage time and temperature influenced enumeration of SPC bacteria. SPC declined slightly at 6°C and increased markedly at 27°C over the 54h holding time. A-MF cultures were extensively overgrown and from 14 to 100% of coliforms recovered were atypical irrespective of holding temperature. In contrast, anaerobic incubation suppressed background non-coliform growth and improved coliform detection. Furthermore, analysis of the data obtained indicated a significant ($p = 0.05$) decrease in recovery of coliforms from water held at 27°C. The coliform isolated was identified as E. cloacae. Similar results (not shown) were obtained when naturally contaminated well water was used to inoculate water from two distribution sites.

M-Endo type media contain selective agents (deoxycholate and lauryl sulfate) which have been shown to inhibit recovery of stressed or injured coliforms (2,15,16) from water. Since anaerobic incubation suppressed overgrowth of MF cultures, a modified m-Endo agar LES medium was formulated in which selective agents were omitted. This medium (LES-LSDC) was compared to the standard formulation for enumeration of coliforms by both A-MF and AN-MF methods. Environmental coliform isolates, including E. cloacae, E. aerogenes, K. pneumoniae and E. coli, were inoculated into high-background drinking water, 100 ml samples were filtered, and MF cultures were incubated aerobically and anaerobically. Results for E. cloacae inoculated water are shown in Figures 3 and 4. Results with the other cultures were similar. A-MF cultures were extensively overgrown in both cases. In contrast, background growth was suppressed on AN-MF cultures and coliforms were isolated with equal facility on both media. Thus non-selective m-Endo agar LES combined with anaerobic incubation may prove useful for recovery of stressed or injured coliforms. This is currently under investigation.

DISCUSSION AND CONCLUSIONS

It is generally accepted that groundwater at moderate depths is of high bacteriological quality and for this reason it is often distributed without treatment or disinfection. Bacteriological analysis of 529 water samples (220 well and 309 distribution) indicated that less than 6% contained total coliforms and 93% contained fewer than 500 cfu/ml SPC bacteria. These results are similar to and extend those of McCabe et al (14). As we observed, however, unusually high rainfall events may result in rapid deterioration of water quality in some wells. Coliforms were not detected in water from randomly selected distribution sites, possibly because of dilution in interconnecting distribution lines. Such wells might, however, serve as

point sources of contamination in isolated distribution systems.

Masking of coliform detection by the MF technique occurred and both frequency and extent of masking were directly related to SPC. Over 13% of the water samples assayed produced overgrowth of MF filters and 49% of those samples having in excess of 500 cfu/ml SPC produced overgrowth. Overgrowth also occurred as a result of storage of water samples at or near ambient temperatures for >24 hours. Detection was unmasked either by placing overgrown filters in LSB and observing for gas formation as previously demonstrated for water samples with high turbidity (10,13) or by verifying atypical colonies picked from overcrowded MF cultures. In the latter case, 55 of 156 atypical colonies derived from 12 overgrown (TN/CG) filter cultures were identified. Thus, overgrowth of MF by non-coliform bacteria may not always inhibit growth of coliforms, but rather sheen formation. Of the atypical colonies verified and identified, 79% were Enterobacter and 21% were Citrobacter (Table 2). These were also the predominant genera of atypical coliforms identified by Evans et al (7) in contaminated drinking water from surface sources. Overgrowth of MF by non-coliform bacteria poses a serious limitation to an otherwise sensitive and accurate technique of assessing the sanitary quality of potable water. During routine testing of finished water, municipal treatment plants, distribution water samples and private water supplies, the occurrence of MF culture overgrowth by non-coliforms was shown to be a substantial problem for 5 to 16% of systems tested (11). Extensive overgrowth of MF necessitates resampling and retesting which, in turn, decreases economy of time, materials and manpower and increases potential health risks (9). Anaerobic incubation of MF cultures markedly reduced background growth and enhanced recovery of coliforms (Tables 5 and 6; Figures 1, 2, 3 and 4). This simple modification of the standard method suppressed growth of aerobic non-coliforms and electively enhanced growth of facultatively anaerobic coliforms. Recent application of this technique to contaminated recreation water resulted in significant increases in recovery of coliforms (Personal Communication, J. Doyle, School of Renewable Natural Resources, University of Arizona). A base cost of $3.32 per standard MF test has been calculated by Geldreich and Kennedy (8). Adjusting this to include cost of expendable (gas-pak, catalyst and anaerobic indicator strips) and non-expendable (anaerobic jar) items would add approximately $0.05 per MF test. Thus anaerobic incubation is cost effective and suitable for monitoring untreated groundwater common to some systems and private water supplies.

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Table 1. Frequency of occurrence of coliforms in potable water samples relative to standard plate count (SPC).

	SPC Range, cfu/ml		
	0 - 100	101 - 500	>500
Number of samples	403	89	37
Samples with Coliforms			
MF Method	16(4)[1]	6(7)	9(24)
MF Range	1-9	1-5	1-82
Samples TN/CG[2]	38(9)	15(17)	18(49)

[1]Relative frequency (percent) of total within range in parentheses

[2]TN, too numerous (>200 countable colonies); CG, confluent growth

Table 2. Identification of coliforms isolated from potable water samples by the MF method.[1]

Isolate	Typical[2] No.	Atypical[3] No.	Total No.	Total %
E. cloacae	5	94	99	39
E. aerogenes	13	1	14	6
E. agglomerans	0	28	28	11
C. freundii	63	33	96	38
C. amalonaticus	1	0	1	0.5
K. pneumoniae	11	0	11	4
E. coli	3	0	3	1.5
TOTALS	96	156	252	100

[1]Coliforms identified by API20E

[2]Typical, sheen

[3]Atypical, no sheen

Table 3. Effect of aerobic and anaerobic incubation on the recovery of coliforms suspended in distilled water at 22°C.

Holding Time (hr)	*K. pneumoniae*		*C. freundii*		*E. aerogenes*		*E. coli*	
	A-MF[1] (100 ml)	AN-MF[2] (100 ml)	A-MF (100 ml)	AN-MF (100 ml)	A-MF (100 ml)	AN-MF (100 ml)	A-MF (100 ml)	AN-MF (100 ml)
0	42/0[3]	40/3	72/1	40/29	41/0	46/0	38/0	44/0
24	55/0	50/0	39/0	23/15	36/0	27/0	37/0	34/0
48	66/0	82/0	22/0	16/5	29/0	24/0	37/0	25/0
72	171/0	174/0	13/0	10/1	20/0	21/0	31/0	22/0

[1]A-MF, aerobic incubation of M-endo agar cultures.

[2]AN-MF, anaerobic incubation of M-endo agar cultures.

[3]No./No., Number of typical (sheen colonies/Number of atypical (no sheen) colonies.

Table 4. Recovery of coliforms from potable water by aerobic (A) and anaerobic (AN) incubation of MF cultures.

Medium	Number of coliforms verified[1] A-MF[2]	AN-MF[3]
M-endo agar	68	151
M-endoagar LES	37	85

[1]Both typical (sheen) and atypical (non-sheen) colonies were verified

[2]A-MF, aerobic incubation

[3]AN-FM, anaerobic incubation

Table 5. Suppression of non-coliform background growth by anaerobic incubation.

Range of Non-coliform Colonies/100 ml Sample	Number and Percent of Water Samples Within Range as Determined by: A-MF[1] No.	%	AN-MF[2] No.	%
0-100	71	55.9	106	83.5
11-100	22	17.3	14	11.0
101-200	12	9.5	2	1.6
>200 (TN/CG)	22	17.3	5	3.9
TOTALS	127	100	127	100

[1]A-MF, aerobic incubation of MF cultures

[2]AN-MF, anaerobic incubation of MF cultures

Table 6. Effect of holding time and temperature on the recovery of coliforms from contaminated well water.

Holding Temperature (°C)	Holding Time (hr)	SPC cfu/ml (x10^3)		A-MF/100 ml[1] T	AT	A-MF Background		AN-MF/100 ml[2] T	AT	AN-MF Background
6	0	5.9		5	4	CG		5	1	1
	24	4.2		4	6	CG		11	0	2
	30	5.5		5	1	CG		8	0	0
	48	3.6		1	9	CG		8	0	0
	54	4.2		8	5	CG		11	2	1
			Total	23	25		Total	43	3	
27	0	6.9		6	1	CG		5	0	0
	24	48		0	4	CG		13	1	0
	30	116		4	3	CG		12	0	0
	48	400		0	3	CG		1	0	0
	54	800		1	3	CG		3	0	0
			Total	11	14			34	1	

[1]A-MF, Aerobic incubation of MF cultures

[2]AN-MF, Anaerobic incubation of MF cultures

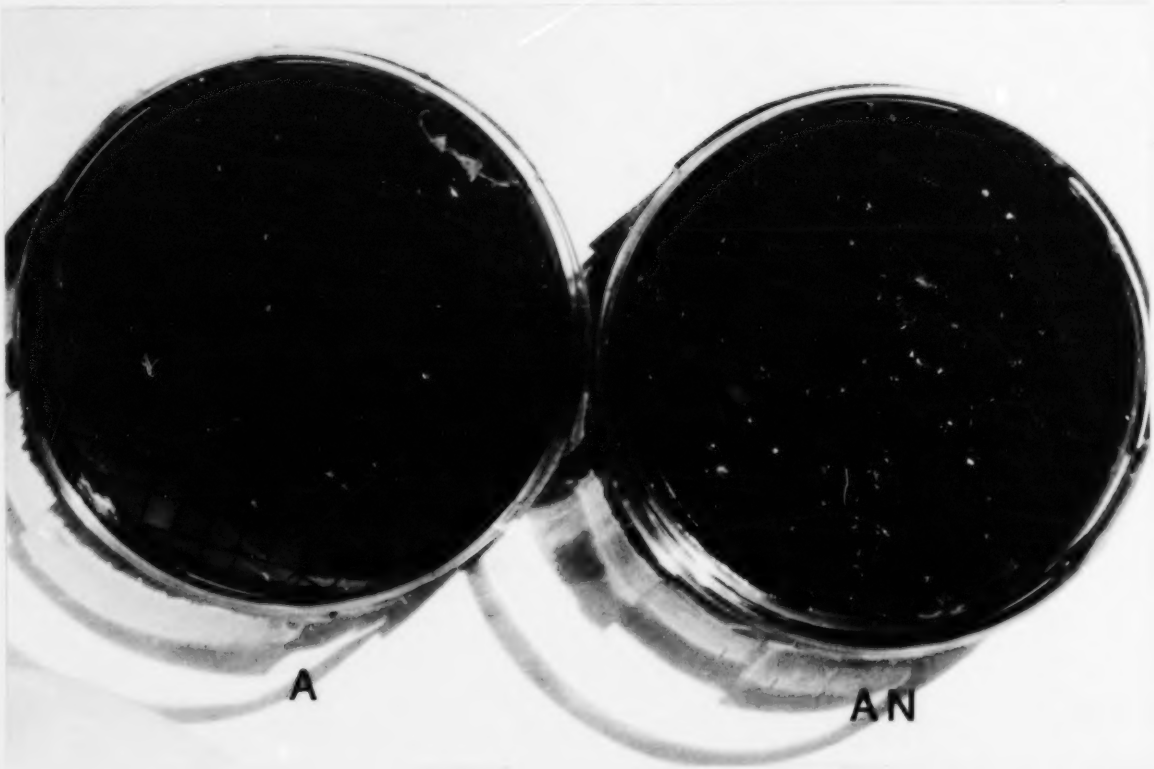

Figure 1. Recovery of coliforms from 100 ml of potable well water by modified anaerobic MF (AN) and standard MF (A) methods.

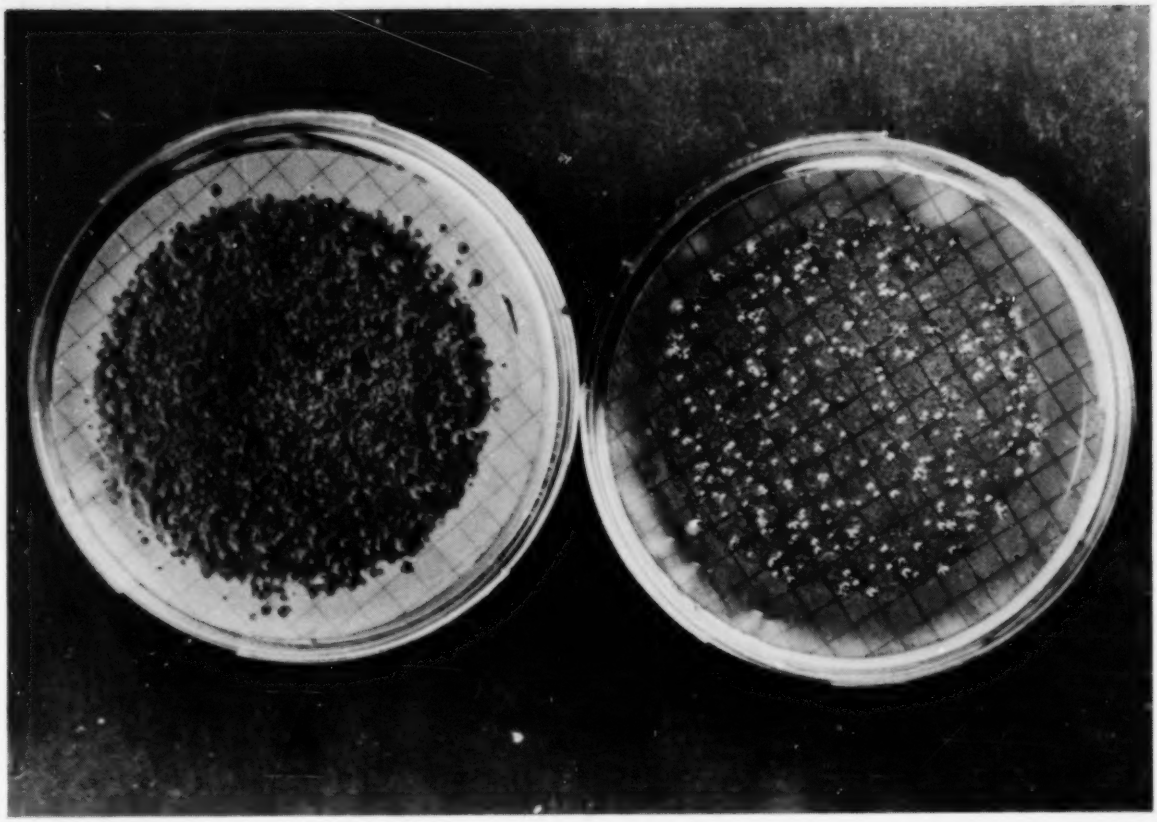

Figure 2. Recovery of coliforms from 100 ml of surface (stream) water by modified anaerobic MF (AN) and standard MF (A) methods.

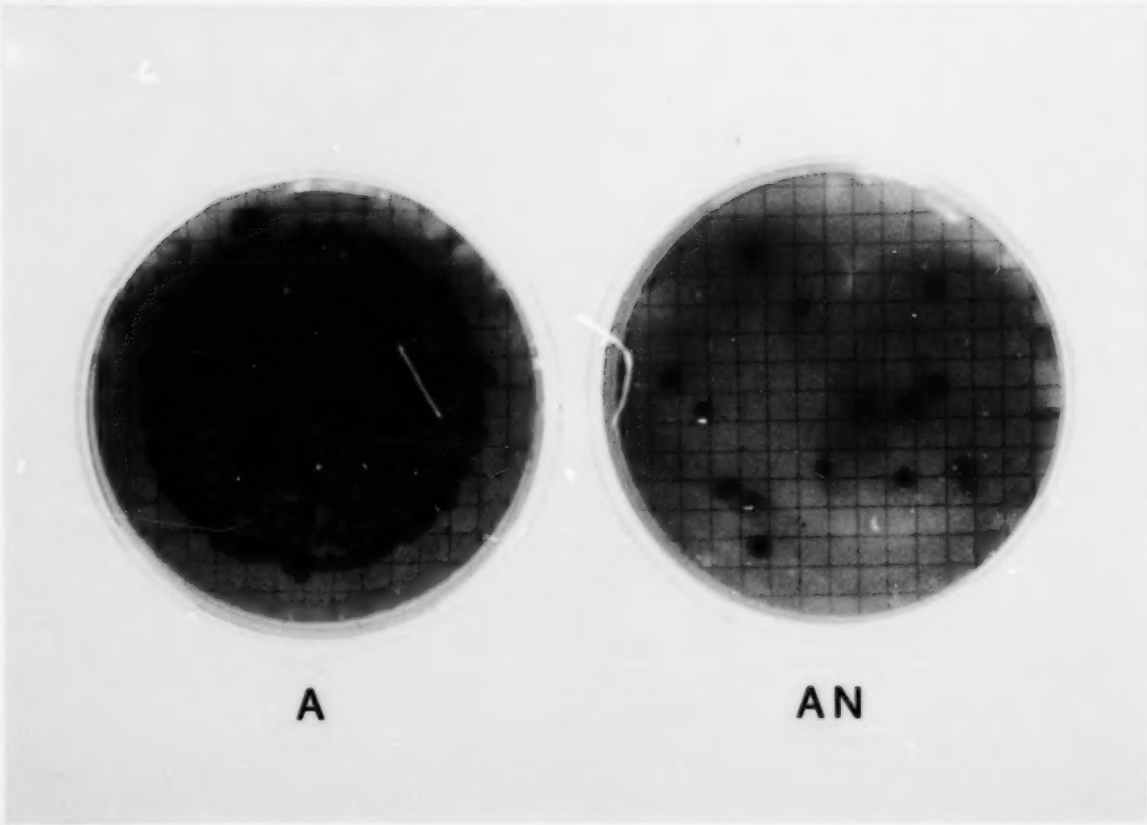

Figure 3. Recovery of E. cloacae from high background drinking water by standard MF (A) and modified anaerobic MF (AN) methods using m-Endo agar LES.

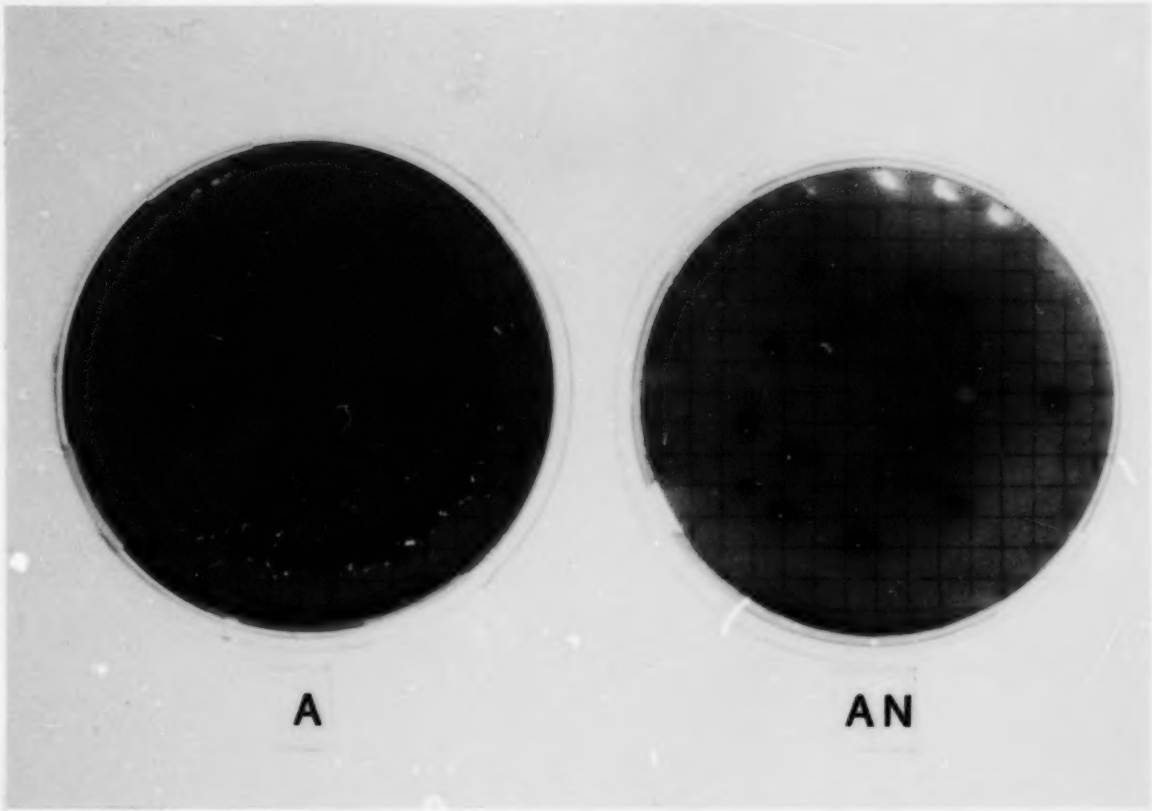

Figure 4. Recovery of E. cloacae from high background drinking water by standard MF (A) and modified anaerobic MF (AN) using m-Endo agar LES minus lauryl sulfate and deoxycholate (LES-LSDC).